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Phosphorylation of Nucleosides with Polyphosphoric Acid

by

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SUMMARY

1. Adenosine, cytidine, guanosine, uridine, deoxycytidine, and thymidine are phosphorylated with polyphosphoric acid (PPA) at temperatures ranging from 0 to 22°C in yields of identified phosphates ranging from 25% to 45%. Deoxyadenosine and deoxyguanosine are not phosphorylated under these conditions.

2. The products formed were separated by ion exchange chromatography on DEAE-cellulose and on AG1 resin. Mono-, di-, tri-, and higher polyphosphates were found.

3. Phosphorylation occurs at the hydroxyl groups of the pentose moiety.

4. These findings are considered relative to the theory of prebiological chemistry.

INTRODUCTION

The use of polyphosphoric acid in polymerizing 2'(3')-cytidylic acid through phosphodiester linkages has been described in previous communications[1-3]. This paper reports the finding that polyphosphoric acid is useful also for phosphorylation of the four ribonucleosides and for the pyrimidine deoxyribonucleosides at low temperatures. These reactions give yields of biologically significant mononucleotides.

MATERIALS AND METHODS

Nucleosides and nucleotides purchased as reference compounds were Calbiochem products of A grade. Polyphosphoric acid, 82-84% P_2O_5, was a Matheson Coleman and Bell product.

Phosphorylation

About 5 mg of nucleoside was dissolved in dist. water and lyophilized. The dried material was quickly mixed with 30-80 mg of polyphosphoric acid with a glass rod and kept from contact with air by tight stoppering. After an appropriate reaction time, the colorless mixture was dissolved in ice cold water and immediately neutralized by adding dilute ammonium hydroxide solution to about 5 ml. Table I represents for each individual nucleoside the reaction time and reaction temperature employed.

Separation on DEAE-cellulose (rA and dT)

The neutralized phosphorylation mixture was placed on a column of DEAE-cellulose (carbonate form, 1 cm^2 x 22 cm). After a water wash which liberated unphosphorylated material, the nucleosidephosphates were eluted with a gradient of triethylammonium bicarbonate (500 ml water + 500 ml 1 M TEAB). The effluents were pooled according to their uv-absorption and repeatedly lyophilized, thus removing excess TEAB.

Separation on AG 1 X 4 (rA, rC, rG, rU, dC, dT)

The neutralized phosphorylation mixture was placed on a column of AG 1 X 4 (formate form, 1 cm^2), and thoroughly washed with water. The elution of nucleosidephosphates was carried out with a linear gradient of formic acid and ammonium formate (and sodium chloride in the case of rA and rG). Table II gives the data on elution technique. The patterns of elution are seen in Figures 3 and 4.

Relative uv-absorbancies of fractions obtained were measured to provide the percent yields listed in Table III.

Electrophoresis*

High-voltage electrophoresis was carried out on Whatman paper No. 1 in a) 0.05 M citric acid and 0.05 M sodium citrate (pH 3.4),

*Savant Instruments, Inc., Hicksville, New York.

15 mA, 1000 V, 2 hrs. b) acetic acid/pyridine/water (10:1:89) (pH 3.5), 40 mA, 1000 V, 3 hrs. Spots were detected in short wave uv-light after drying.

Thin-layer chromatography**

a) MN cellulose powder 300[4], 0.5 mm, tert.-amyl alcohol/ formic acid/water (5:2:1), 1 hr. b) MN cellulose powder 300 DEAE[5], 0.5 mm, 0.05 N HCl, 1 hr. c) MN cellulose powder 300 PEI[6], 0.5 mm, 2 min. with 0.2 N LiCl, 15 min. with 0.8 N LiCl, 40 min. with 1.2 N LiCl. Spots were visible under short wave uv-light after drying.

Rf-value comparisons on AG 1 X 4 columns

a) Purified fractions (about 0.5 mg) of the phosphorylation of adenosine and of thymidine obtained by chromatography on DEAE-cellulose were repurified on a second DEAE-cellulose column and mixed with purchased material (about 0.5 mg). This solution was run through a column of AG 1 X 4 in a linear gradient. Only single peaks could be observed.

b) For establishment of identity small amounts of purchased nucleotides (0.5-1.0 mg) were dissolved in the neutralized phosphorylation mixture of each nucleoside and run through a column of AG 1 X 4 in a linear gradient.

**Brinkmann Instruments, Inc., Westbury, New York.

RESULTS

In Figs. 1 and 2 curves of the uv-absorption of phosphorylation products (adenosine and thymidine) eluted from DEAE-cellulose with a TEAB gradient are shown. The first peak, representing starting material, was eluted with water, whereas the subsequent fractions were stripped from the column with gradually increasing concentrations of TEAB. In Fig. 1 peaks 1, 2, 3, 4, and 5 represent predominantly adenosine, 5'-AMP, 2'(3')-AMP, 5'-ADP, and 5'-ATP, respectively. Material of peak 5 gave a positive firefly lantern test[7]. Peaks 6, 7, and 8 are unidentified adenosinephosphates as established by uv-spectroscopy. In Fig. 2 peaks a, b, c, and d are predominantly thymidine, 5'-dTMP, 5'-dTDP, and 5'-dTTP, respectively. Peaks e, f, and g are unidentified thymidinephosphates.

Further fractionations of phosphorylation products were carried out by chromatography on AG 1 X 4, with a linear gradient of formic acid/ammonium formate (and sodium chloride). Fig. 3 shows the fractionations of phosphorylation products of adenosine, cytidine, guanosine, and uridine, Fig. 4 those of deoxycytidine and thymidine. The results from deoxyadenosine and deoxyguanosine are not described since their phosphorylation with polyphosphoric acid yielded only dark brown or black tars in which significant amounts of nucleotides were not found. Fractionation was followed by checking the uv-absorbancies of the effluent. Substantial amounts of nucleosidephosphates are seen to be formed. The relative yields, expressed as percent, are listed in Table III.

Further tests of identity of each of the 6 nucleosides examined were made by adding purchased material to the mixture of phosphorylation product and percolating the solution through a column of AG 1 X 4, with application of the above mentioned gradients. As an example, the phosphorylation of uridine may be considered. Figs. 5 and 6 represent the original phosphorylation mixture and the phosphorylation mixture plus 5'-UDP, respectively. No other peaks can be seen; however, a marked increase of the already existing 4th peak is observed, with the original symmetry. This result indicates identity of the 5'-UDP formed with authentic 5'-UDP.

DISCUSSION

Polyphosphoric acid has been used in several studies of the phosphorylation of nucleosides and their derivatives. Hall and Khorana[8] added orthophosphoric acid and phosphorus pentoxide to 2',3'-O-isopropylidene uridine or to 2',3'-O-isopropylidene cytidine and heated the mixture at 60°. The protective group was removed by acidic hydrolysis at 100°, thus giving pyrimidine nucleoside-5'-monophosphates in high yields. A similar procedure was followed by Michelson[9], who used 2',3'-O-benzylidene uridine or 2'-3'-O-benzylidene cytidine, hydrolyzed at 100°, and isolated nucleoside-5'-monophosphates in good yield. With free uridine and prolonged reaction time, 2'(3'), 5'-uridine diphosphate was found. This method of phosphorylation with polyphosphoric acid has also been used to prepare P^{32}-labeled nucleotides[10].

The experiments described in this paper show that substantial amounts of nucleoside mono-, di-, tri-, and poly- phosphates can be formed and identified. In contrast to the results cited above, no splitting of acid-labile glycosidic bonds of purine ribonucleosides is encountered when low temperatures are used during the phosphorylation procedure and during the subsequent neutralization step. Additionally, no polyphosphate group is cleaved and degraded to the monophosphate state under the conditions employed here. The phosphorylation of purine deoxyribonucleosides with polyphosphoric acid, however, even at temperatures below 0°C, yields dark brown or black tars. Traces of nucleotides are found, in the case of deoxyguanosine only.

By comparing the yields of adenosine-phosphates prepared by the action of polyphosphoric acid on one hand, and of pyrophosphoric acid on the other (Table III), one observed that the overall yield of nucleotides, especially of di-, tri-, and higher poly- phosphates, is substantially greater in the case of polyphosphoric acid. This leads to the inference that intact chains of polyphosphoric acids are linked to sugar hydroxy groups, seemingly displaying a slight preference for the stereochemically favored 5'-hydroxy group. The relative reactivity of pyrophosphoric acid is of interest since pyrophosphoric acid is a principal constituent of polyphosphoric acid[11].

Polyphosphoric acid has been used in several studies[1-3,11,12], carried out with the purpose of disciplining the theory of prebiological chemistry. The now demonstrated ease and simplicity of phosphorylation of nucleosides with polyphosphoric acid supports the plausibility of this reaction in the prebiotic synthesis of nucleotides, especially the triphosphates. Experiments along similar lines have been performed by Ponnamperuma[13], who showed that monophosphates of adenosine, cytidine, guanosine, uridine, and thymidine are easily synthesized in substantial yields by

heating nucleosides at 150°C with either orthophosphoric acid or its monobasic salts. Ponnamperuma observed a marked decline of yield with dibasic salts and a more pronounced decrease with tribasic salts of orthophosphoric acid. By combining the inference from the results of Ponnamperuma with those described in this paper, one may conclude that 1) the formation of nucleoside di- and tri-phosphates is facilitated with "preformed" polyphosphoric acid and 2) phosphorylation takes place more easily with the higher states of protonation of the phosphoric acids.

The utility of phosphoric acid[11] and of the salts of phosphoric acid has also been demonstrated with calcium phosphates and sodium phosphates in the polymerization condensation of α-amino acids[14]. Taken in conjunction with the formation of the four ribonucleoside phosphates and the pyrimidine deoxyribonucleoside phosphates, with other reactions[12], and with the known significance of phosphates in biochemistry[15], such reactions point increasingly to the significance of a primordial phosphoric medium[12].

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TABLE I

CONDITIONS OF PHOSPHORYLATION OF NUCLEOSIDES WITH PPA

	rA	rC	rG	rU	dC	dT	rA*
Time (in hrs.)	1-2	3-6	1-2	0.5	0.5	2-4	1.0
Temperature	5°	5°	22°	22°	22°	5°	17°

*Molten phyrophosphoric acid was used instead of PPA.

TABLE II

CONDITIONS OF FRACTIONATION OF PHOSPHORYLATED NUCLEOSIDES

	column		mixing vessel			reserve vessel		
	in cm	ml	H_2O	N HCOOH	ml	N HCOOH	N $HCOONH_4$	N NaCl
rA	26	500		0.25	500	1.0	2.0	0.3
rC	30	300	"		300	0.5	2.0	
rG	16	400		1.0	400	4.0		0.6
rU	27	350	"		350	1.0	4.0	
dC	28	300	"		300	1.0	4.0	
dT	27	350	"		350	1.0	4.0	

TABLE III

RELATIVE YIELDS* IN PHOSPHORYLATION OF NUCLEOSIDES WITH POLYPHOSPHORIC ACID

	rA**	rC	rG	rU	dC	dT**	rA***
unphosphorylated material	44.8	21.4	48.0	32.0	31.2	50.2	85.0
5'-MP	7.4	9.0	11.7	8.9	9.2	14.6	6.6
2'-MP	3.2	16.7	14.9	13.4			1.9
3'-MP	6.7						3.6
5'-DP	9.6	9.1	9.9	10.1	10.1	12.3	2.9
5'-TP	5.7	5.4	3.8	11.8	7.6	8.5	
unidentified	22.7	38.4	11.5	23.8	41.9	14.2	

*No correction has been made for differences in ε max of nucleosides and nucleosidephosphates.

**Average of 5 measurements; others represent single determinations.

***Phosphorylation was carried out with molten pyrophosphoric acid.

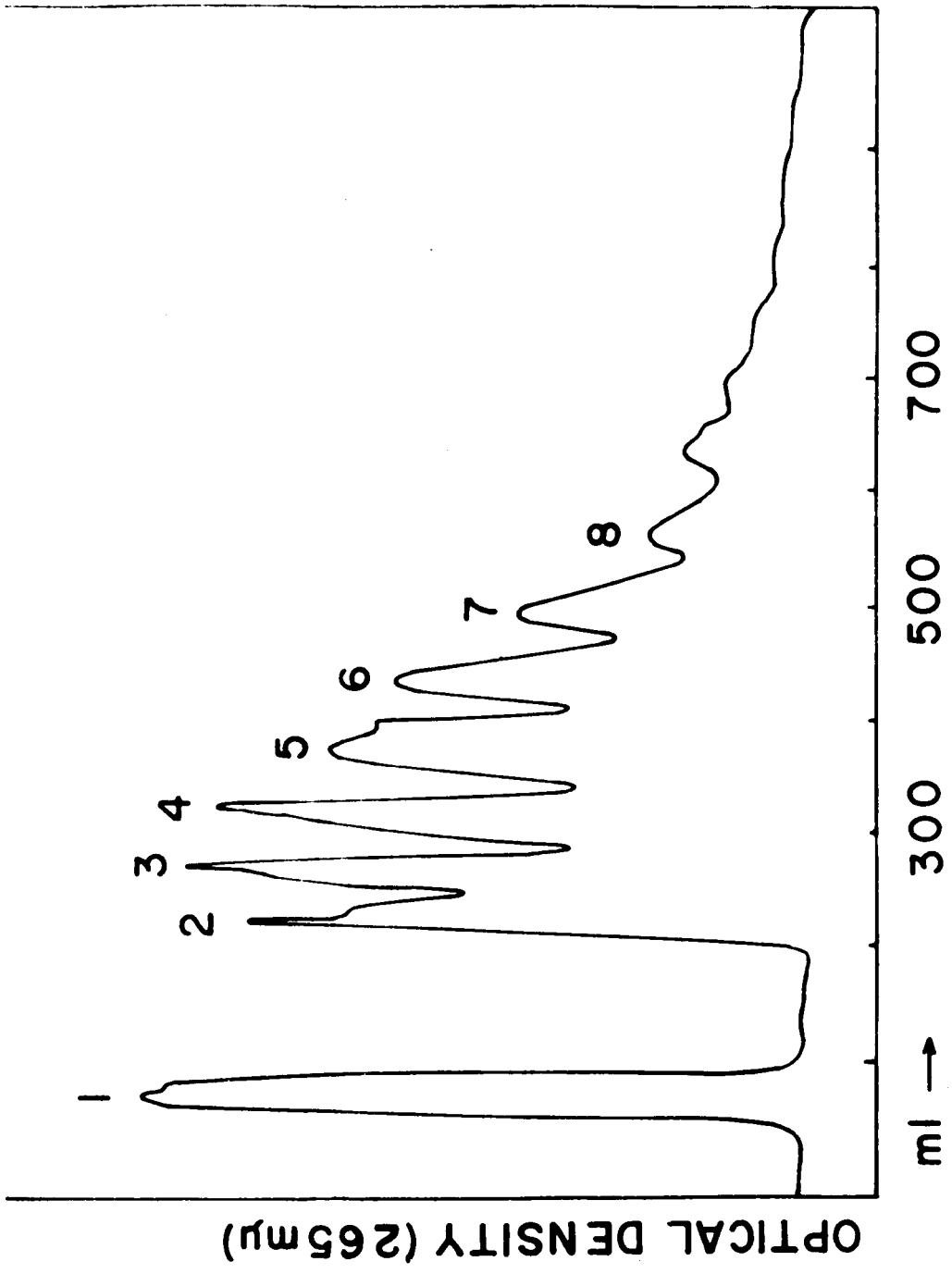


Fig. 1. Fractionation of phosphorylation products of adenosine, DEAE-cellulose, TEAB gradient.

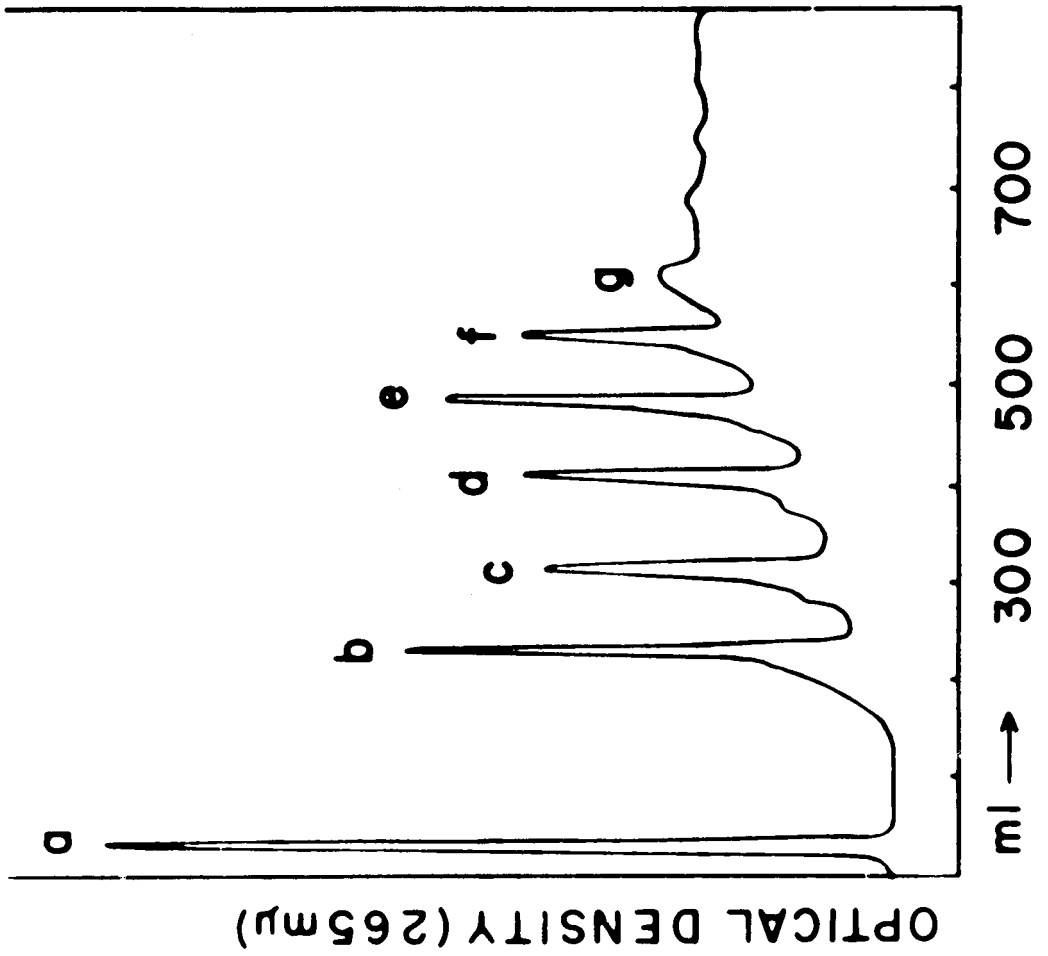


Fig. 2. Fractionation of phosphorylation products of thymidine, DEAE-cellulose, TEAB gradient.

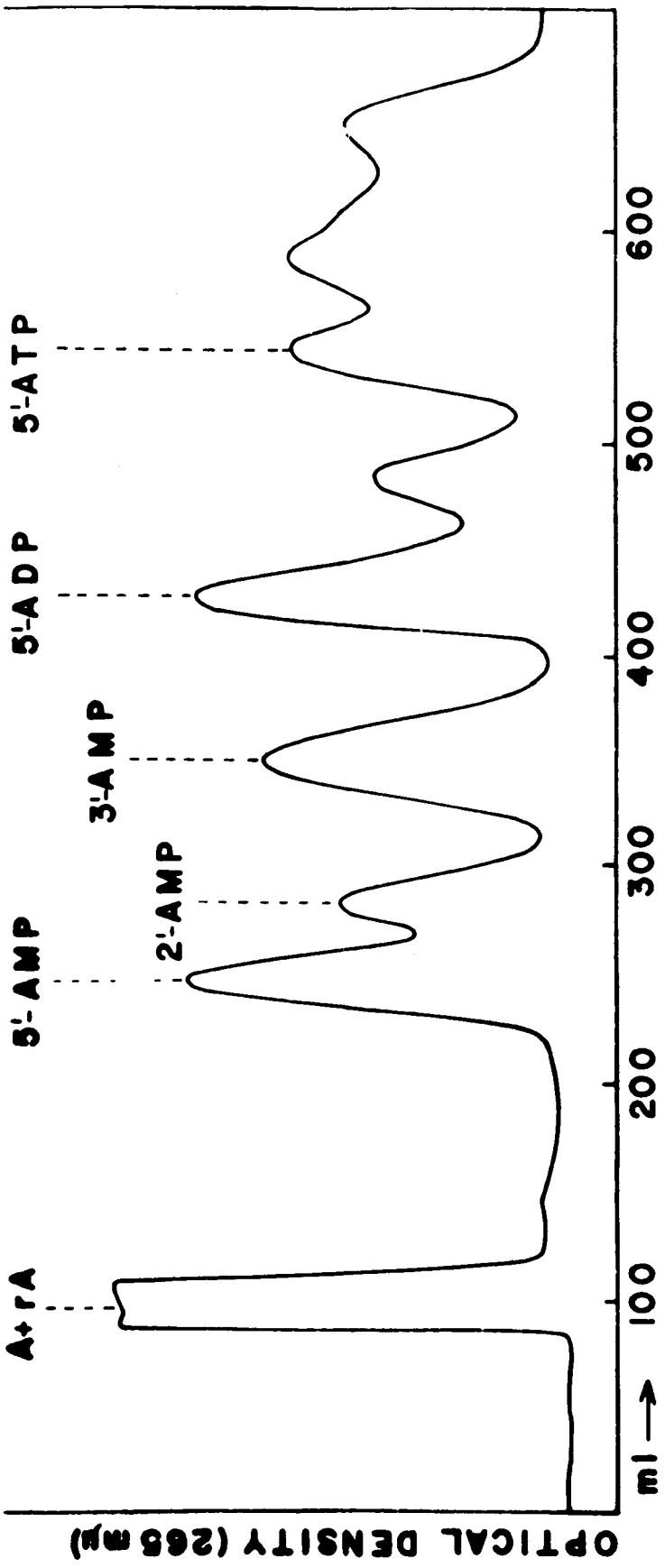
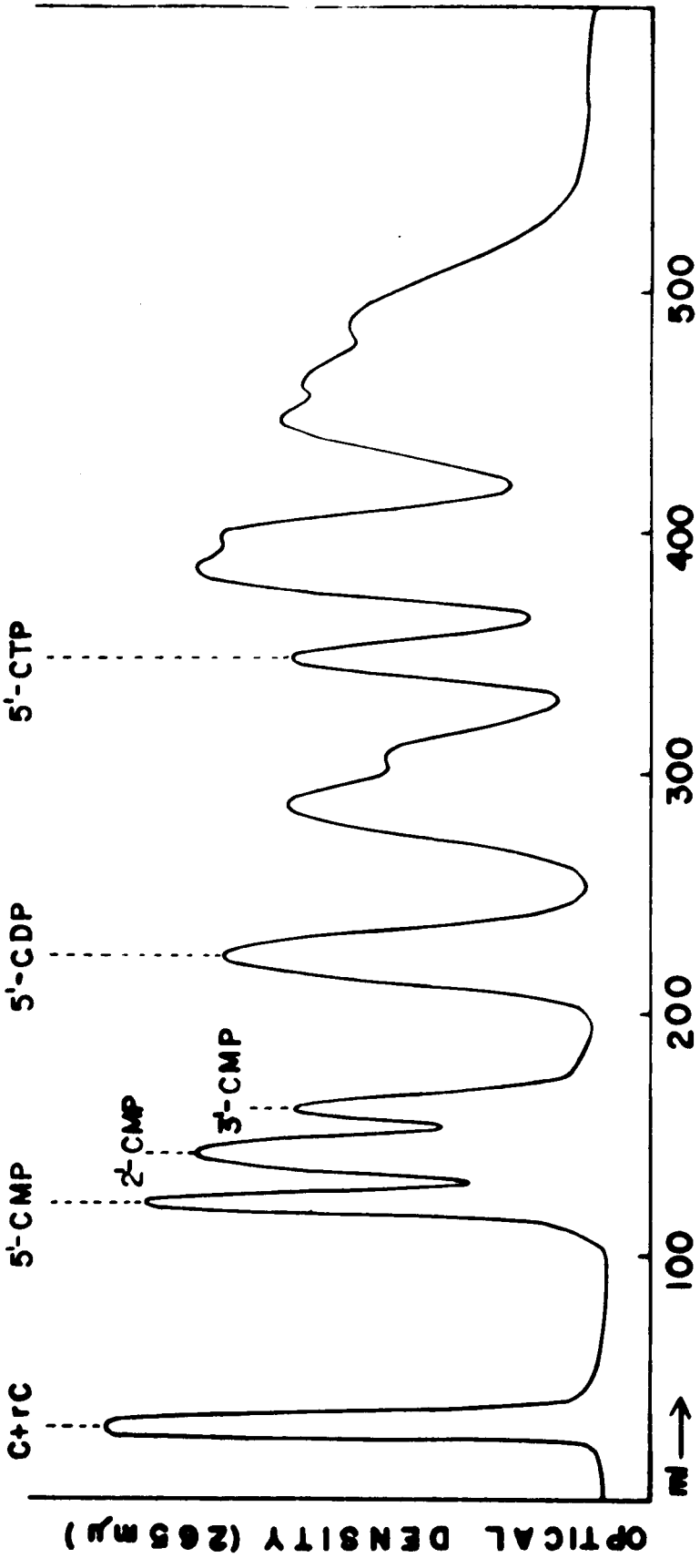


Fig. 3. Elution pattern of phosphorylation products of adenosine, cytidine, guanosine, and uridine. AG 1 X 4, formic acid/ammonium formate gradient.



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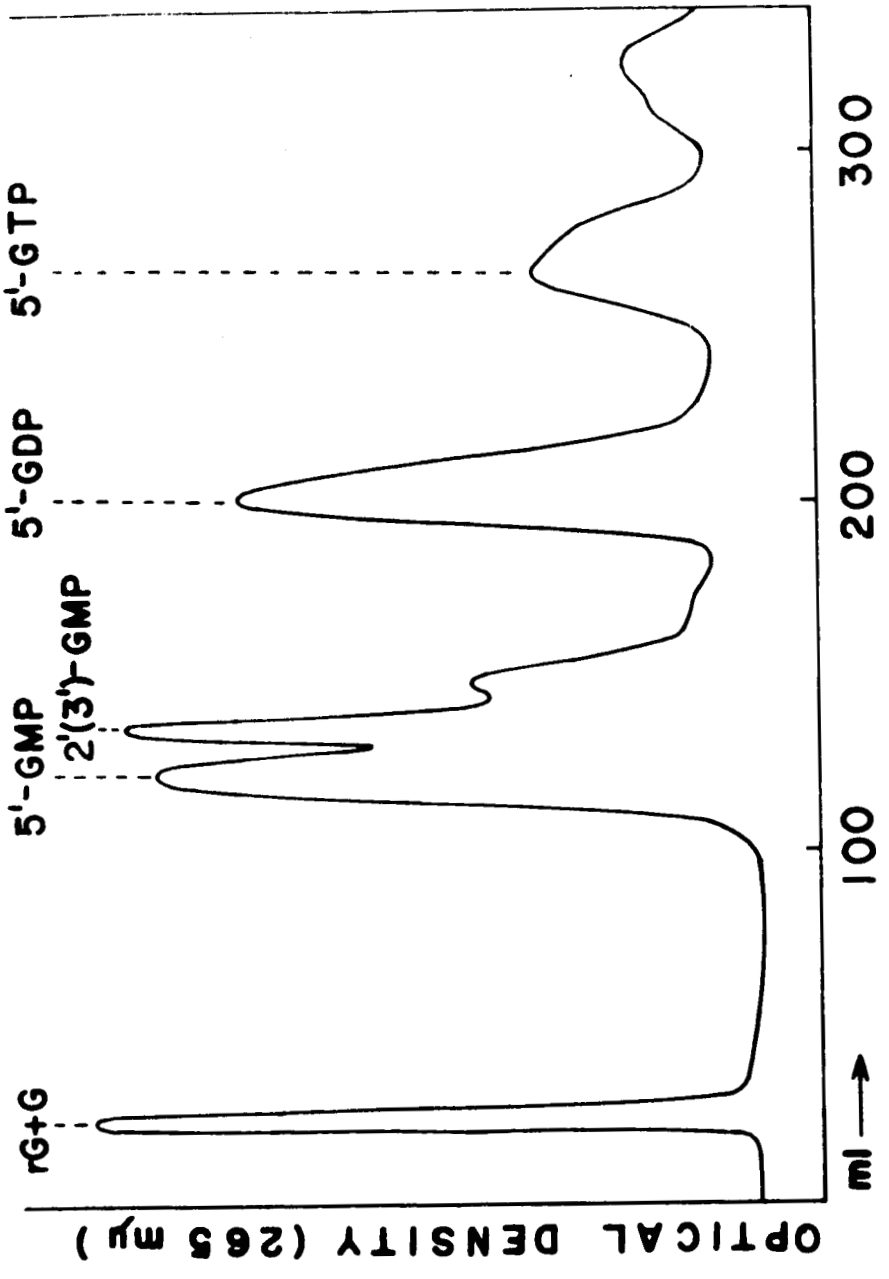

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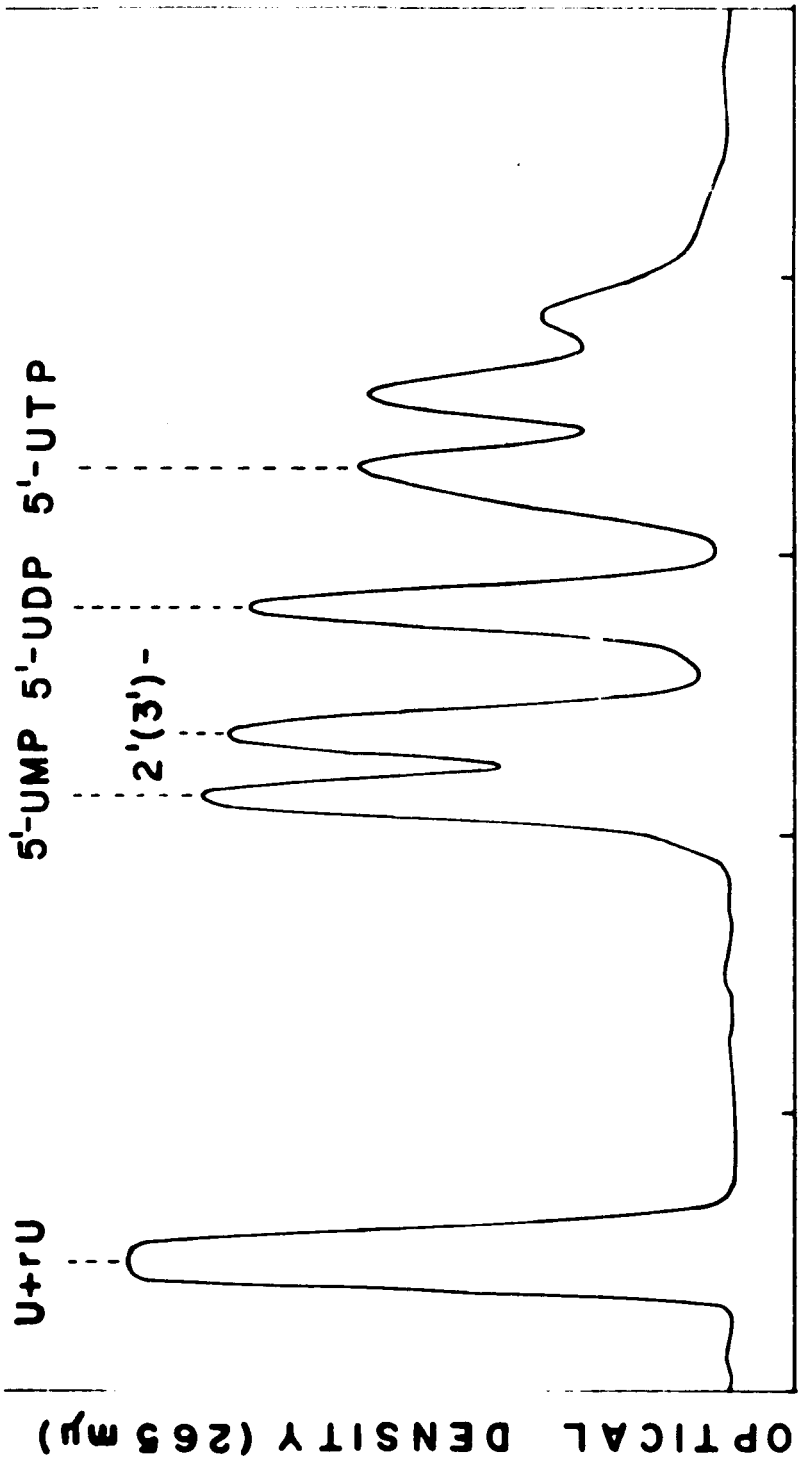

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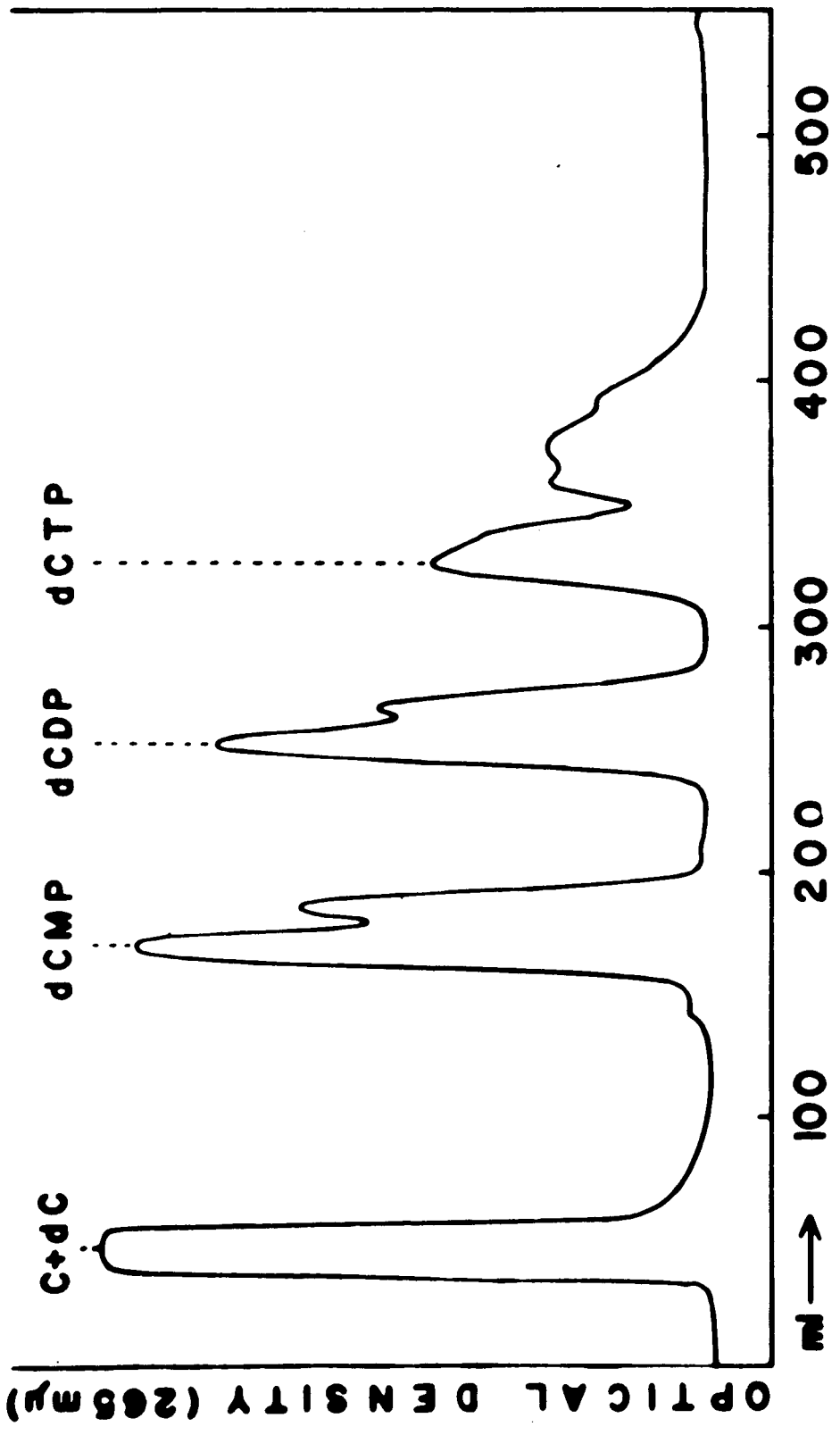

Fig. 4. Elution pattern of phosphorylation products of deoxycytidine and thymidine. AG 1 X 4, formic acid/ammonium formate gradient.

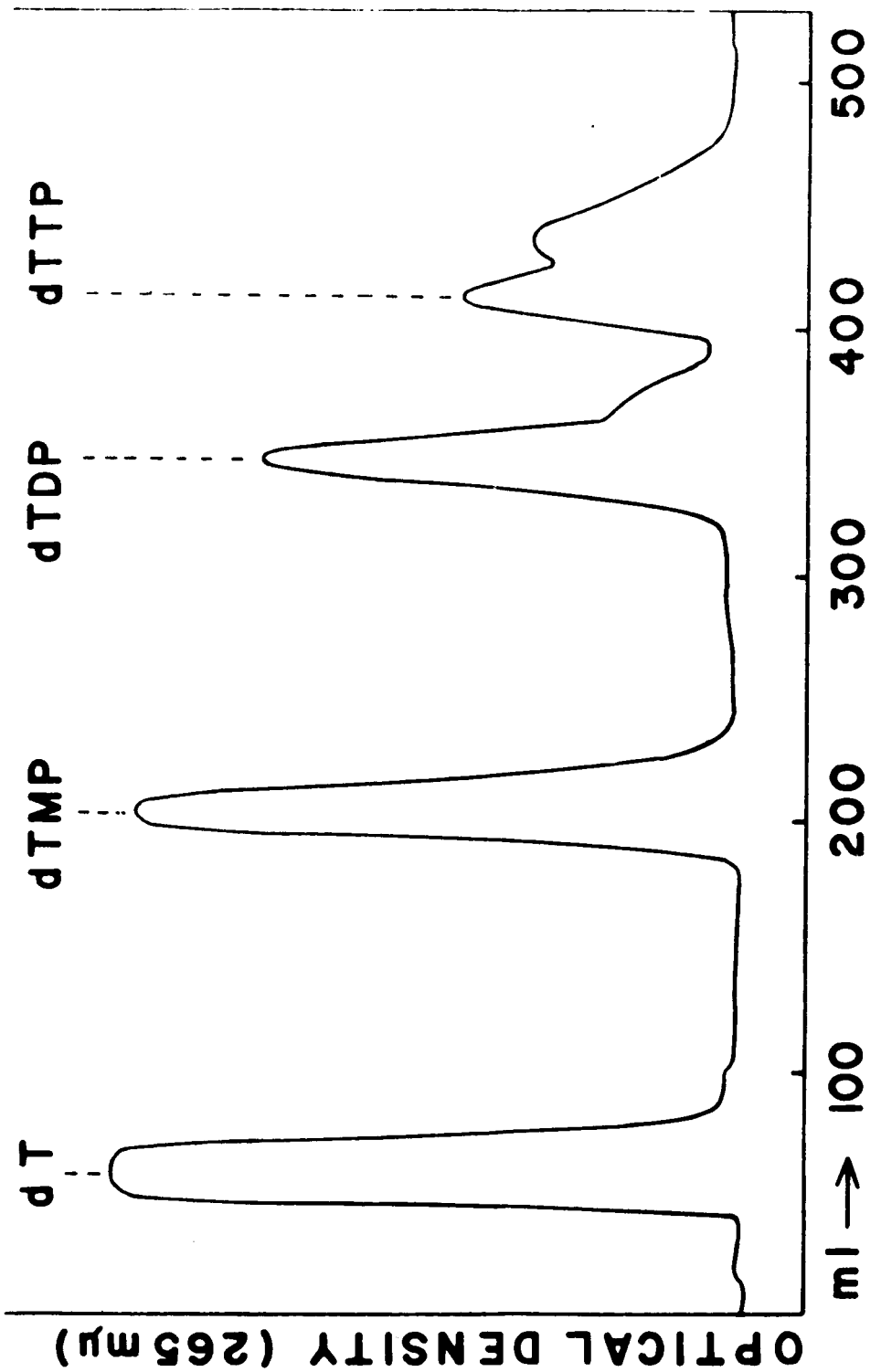

Fig. 4. Elution pattern of phosphorylation products of deoxycytidine and thymidine. AG 1 X 4, formic acid/ammonium formate gradient.

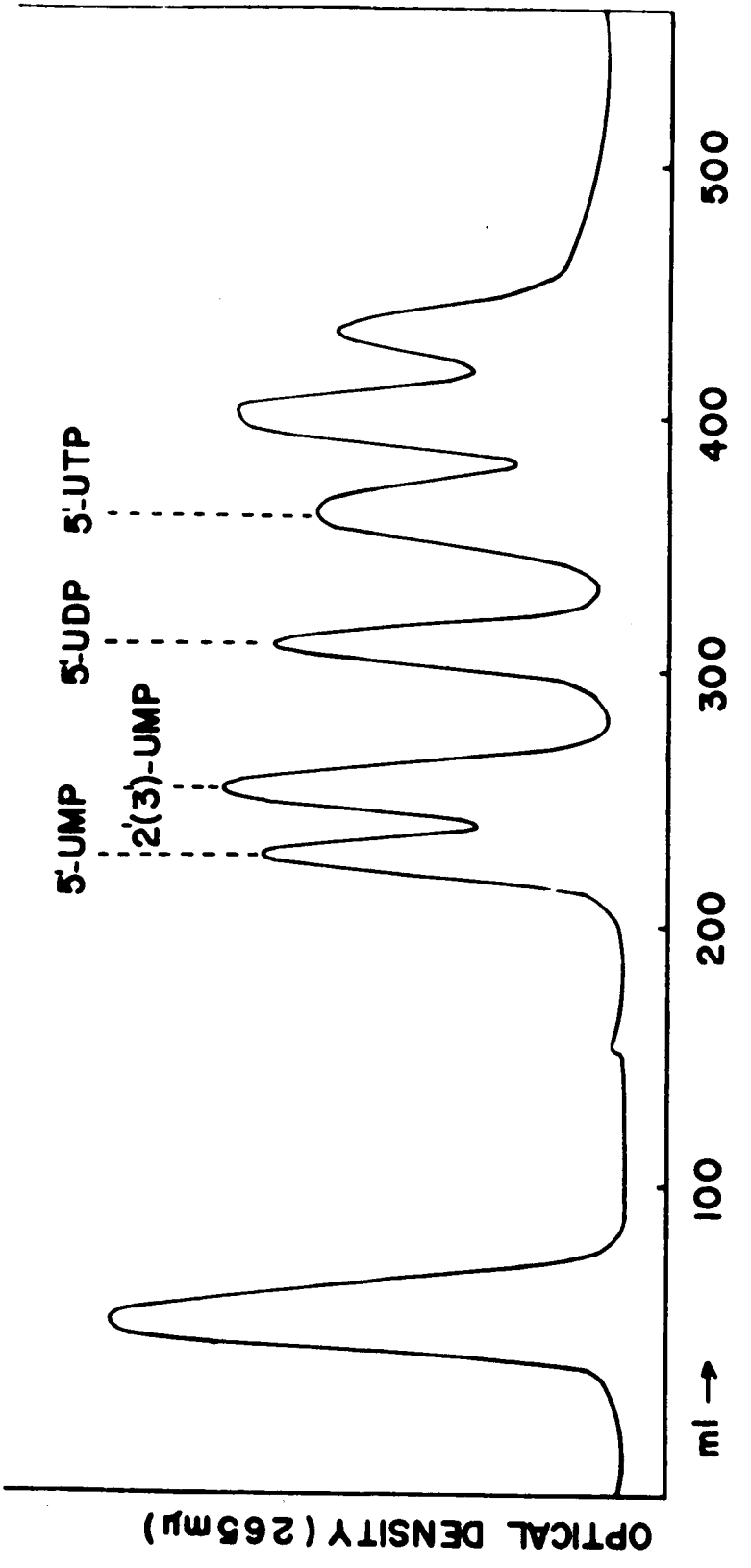

Fig. 5. Elution pattern of phosphorylation products of uridine. AG 1 X 4, formic acid/ammonium formate gradient.

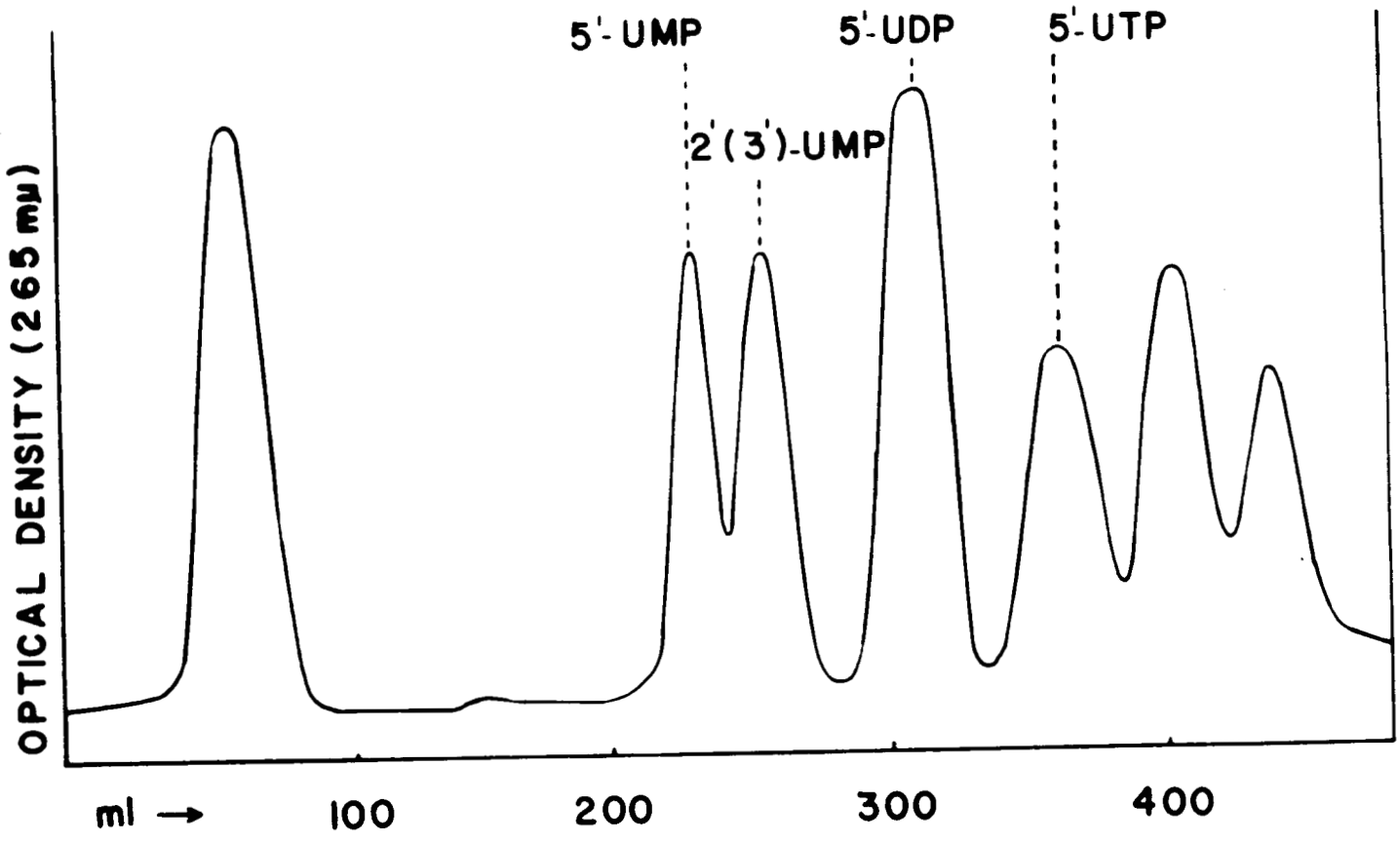

Fig. 6. Elution pattern of phosphorylation products of uridine plus authentic 5'-UDP. AG 1 X 4, formic acid/ammonium formate gradient.